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TITLE: Methods for screening for antibiotics

BSPR:
Resistance to currently available antibiotics has created a need for new antibiotic agents. In the United States alone, 19,000 hospital patients die each year due to nosocomial (hospital-acquired) bacterial infections (Service, R., 1995, Science 270:724-727). These infections, caused by organisms such as Staphylococcus aureus, Pseudomonas aeruginosa, Enterococcus faecium and Enterococcus faecalis, have become increasingly resistant to currently approved antibiotics. For example, significant clinical problems include methicillin-resistant strains of S. aureus, which are resistant to all current antibiotics except vancomycin (a drug of last resort because of severe side effects), and a vancomycin-resistant strain of E. faecium enterococci which is now found world-wide. The occurrence of vancomycin-resistant enterococci isolated from nosocomial infections rose from 0.4% to 13.6% in the relatively short time span from 1989 to 1993 (Tenover, F. C. and Hughes, J. M., 1996, JAMA 275(4):300-304) (reporting statistics from the Centers for Disease Control and Prevention). Even community-acquired organisms such as Streptococcus pneumoniae are increasingly resistant to antimicrobial agents, with a significant number of isolates being resistant to penicillin and extended-spectrum cephalosporins. Id.

BSPR:
The emergence and spread of resistant bacterial organisms are primarily caused by acquisition of drug resistance genes, resulting in a broad spectrum of antibiotic resistance (e.g., extended-spectrum cephalosporin-resistant mutant .beta.-lactamases found in several bacterial organisms). Genetic exchange of multiple-resistance genes, by transformation, transduction and conjugation; combined with selective pressures in settings such as hospitals where there is heavy use of antibiotic therapies, enhance the survival and proliferation of antimicrobial agent-resistant bacterial strains occurring by, e.g., spontaneous mutants. Id. Although the extent to which bacteria develop resistance to antimicrobial drugs and the speed with which they do so vary with different types of drugs, resistance has inevitably developed to all antimicrobial agents (Gold and Moellering, Jr., 1996, New Eng. J. Med., 335(19):1445-1453).

BSPR:
For example, sulfonamides (or sulfa drugs), the first important antimicrobial agents identified, are actually antimetabolites and riot antibiotics. Sulfanilamide, one of the sulfonamide class drugs, is a structural analog of para-aminobenzoic acid ("PABA"). The mode of action of sulfanilamide was unknown until it was discovered that PABA is required for the synthesis of the essential vitamin, folic acid. Folic acid synthesis is required for bacterial growth since bacteria are not capable of folic acid uptake. Sulfonamides inhibit the bacterial synthesis of folic acid by acting as competitive inhibitors of PABA. For humans, folic acid is also an essential vitamin, but unlike bacteria, humans are capable of uptake of folic acid and can obtain the vitamin through diet. As a result, bacteria, but not humans, are vulnerable to sulfa drugs which inhibit folic acid synthesis. In the sulfa class alone, thousands of chemically modified derivatives have been studied with about 25 of them still in use.

BSPR:
Similarly, much has been learned about peptidoglycan synthesis since the discovery of the penicillin and cephalosporins (peptidoglycan is the critical component in maintaining the shape and rigidity of both Gram positive and Gram negative bacterial organisms). Therefore the discovery of new classes of drugs can broaden the general understanding of bacterial physiology as well as provide for new

antibacterial chemotherapeutics.

BSPR:
The present invention provides methods and compositions for identifying compounds that are capable of causing the accumulation of ppGpp in bacteria.

BSPR:
Test compounds are obtained from a wide variety of sources including collections of natural products in the form of bacterial, fungal, plant and animal extracts; and synthetical chemical libraries. Numerous means known in the art are available for the random, directed and combinatorial synthesis of a wide variety of chemical structures. In addition, natural products or known antibiotic compounds may be subjected to random or directed chemical modifications to produce derivatives and structural analogs for use as test compounds in the invention. Usually various predetermined concentrations are used for screening such as 0.001 .mu.M, 0.01 .mu.M, 0.1 .mu.M, 1.0 .mu.M, 10 .mu.M, and 100 .mu.M.

BSPR:
The test cells may be cultured under standard conditions of temperature, incubation time, optical density, plating density and media composition corresponding to the nutritional and physiological requirements of the bacteria. However, conditions for maintenance and growth of the test cell may be different from those for assaying candidate test compounds in the screening methods of the invention. Modified culture conditions and media are used to facilitate detection of the expression of a reporter molecule. Any techniques known in the art may be applied to establish the optimal conditions.

BSPR:
Depending on the screening technique and nature of the signal used to assay the reporter gene expression, a reporter regimen can be used to aid directly or indirectly the generation of a detectable signal by a reporter molecule. A reporter regimen comprises compositions that enable and support signal generation by the reporter, such as substrates and cofactors for reporter molecules that are enzymes; e.g., lactose-tetrazolium medium. Such compositions are well known in the art. Components of a reporter regimen may be supplied to the test cells during any step of the screening assay.

DEPR:
The assay strain is plated as a lawn on solid medium containing AT, for example, at a concentration of 15 mM. Test compounds are then applied to the medium in wells or on disks. Paradoxical growth is determined by visually comparing growth around the well or disk containing the test compound to growth in control areas which are free of the test compound. Comparison of test and control areas is done at the same time point. Compounds that cause ppGpp accumulation show a ring of enhanced growth at the periphery. Compounds which cause ppGpp accumulation, such as NaN.sub.3 (sodium azide) (Murray and Bremer, 1996, J. Mol. Biol. 259:41-57), or 1,10-phenanthroline are included in the assay as a control since the test strain is not expected to grow on the medium in the absence of a ppGpp degradase inhibitor.

DEPR:
In this embodiment, the assay strain is an E. coli strain which is a relA mutant with reduced PSI synthetase activity but with normal or wildtype PSII synthetase activity and degradase activity (spoT.sup.+). This strain also contains the reporter gene lacZ under the control of a promoter which is negatively controlled by ppGpp. The strain is plated as a is lawn on solid lactose tetrazolium medium (50 .mu./ml 2,3,5-triphenyl-2H tetrazolium chloride), and compounds are added in wells, as described in Section 5.4.1. The cells added to the agar plate are actively growing (log phase) but dilute enough to avoid crowding of cells and inaccurate results. Colony color around the well or disk containing test compound is compared to control areas of the plate that are free of test compound. Comparison of test and control areas is done at the same time point. Compounds that cause ppGpp accumulation are expected to show a ring of red growth around the well or disk. In other lactose differential medium, results will be analogous but the positive and negative readout will depend on the indicator of lactose fermentation. Compounds that cause ppGpp accumulation, such as NaN.sub.3, 1,10-phenanthroline or picolinic acid is included in the assay as a positive control. At high concentrations NaN.sub.3 (sodium azide) is toxic, and therefore there is a zone of inhibited bacterial growth close to the disk. At some distance

from the disk, a ring of red growth is seen, indicating that at some lower concentration NaN.sub.3 is allowing ppGpp accumulation and the cells have become lac.sup.-.

DEPR:
Ribosomes with active ppGpp synthetase I (relA) are prepared from a strain of E. coli that carries a plasmid encoding the relA gene under the control of an inducible promoter. These ribosomes are then combined with GTP and ATP, and the relA protein synthesizes both ppGpp and pppGpp. CF3120 cells are grown in Luria broth containing 100 .mu.g/ml ampicillin to an A.sub.600 of 1.5. relA expression is then induced by the addition of IPTG to a final concentration of 1 mM. Cells are incubated for 1 hour, then harvested by centrifugation. The cell pellet is washed in ribosomal buffer (50 mM Tris acetate [pH 8.0], 15 mM Mg acetate, 60 mM potassium acetate, 27 mM ammonium acetate, 1 mM DTT and 0.2 mM EDTA) and the resulting cell pellet is stored at -70.degree. C. The frozen cell pellet is resuspended in 2 volumes (w/v) of ribosomal buffer then cells are lysed by French press. The lysate is centrifuged at 11,000.times.g for 40 min at 4.degree. C. The supernatant is centrifuged at 30,000 rpm in a Beckman Ti65 (or equivalent) for 4 hrs at 4.degree. C. The resulting pellet of ribosomes and membranes is combined with 2.5 volumes of cold ribosomal buffer, transferred to a beaker and stirred slowly overnight at 4.degree. C. The solution is then centrifuged at 7,500.times.g for 15 min at 4.degree. C. to remove undissolved debris. The supernatant is removed and ribosomal buffer is added to bring the suspension to 4.times. (w/v) with respect to the original weight of the cells. A 5 ml cushion of 40% sucrose in ribosomal buffer is placed in a 30 ml ultracentrifuge tube then the ribosomal suspension is carefully layered on top, filling the tube. The preparation is centrifuged at 32,000 rpm in a Beckman Ti65 (or equivalent) for 4 hrs at 4.degree. C. The supernatant is discarded and the pellet is transferred to a beaker containing a minimal volume of cold ribosomal buffer. The mixture is stirred at 4.degree. C. until resuspended then stored by dropping drops into a beaker filled with liquid nitrogen. The drops freeze and can be stored in vials at -70.degree. C. Just before use, drops are transferred to a tube and thawed on ice.

DEPR:
In yet another embodiment, the invention provides novel antibiotic agents discovered by the methods described above. These antibiotic agents are capable of causing ppGpp accumulation in a bacterial cell, leading to downregulation of rRNA synthesis, and ultimately to a reduction or inhibition of bacterial growth. These agents may, for example, act by enhancing PSII activity, and/or inhibiting ppGpp degradase activity, and are expected to be effective in a variety of species of bacteria, including infectious pathogenic bacteria. The invention also includes novel pharmaceutical compositions which comprise antibiotic agents discovered as described above formulated in pharmaceutically acceptable formulations.

DEPR:
The antibiotic compounds identified by methods of the invention may be formulated into pharmaceutical preparations for administration to animals for treatment of a variety of infectious diseases. Compositions comprising a compound of the invention formulated in a compatible pharmaceutical carrier may be prepared, packaged, labelled for treatment of and used for the treatment of the indicated infectious diseases caused by microorganisms, such as those listed infra in Section 5.9.3.

DEPR:
For oral administration, the pharmaceutical preparation may be in liquid form, for example, solutions, syrups or suspensions, or may be presented as a drug product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, or fractionated vegetable oils); and preservatives (e.g., methyl or propyl-p-hydroxybenzoates or sorbic acid). The pharmaceutical compositions may take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding agents (e.g., pregelatinized maize starch, polyvinyl pyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets

may be coated by methods well-known in the art.

DEPR:
For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

DEPR:
The compounds may be formulated for parenteral administration by injection, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampules or in multi-dose containers, with an added preservative. The compositions may take such forms as suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile pyrogen-free water, before use.

DEPR:
The antibiotic compounds may also be formulated in rectal compositions such as suppositories or retention enemas, e.g., containing conventional suppository bases such as cocoa butter or other glycerides.

DEPR:
In addition to the formulations described previously, the antibiotic compounds may also be formulated as a depot preparation. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. Thus, for example, the antibiotic compounds may be formulated with suitable polymeric or hydrophobic materials (for example, as an emulsion in an acceptable oil) or ion exchange resins, or as sparingly soluble derivatives, for example, as a sparingly soluble salt. Liposomes and emulsions are well known examples of delivery vehicles or carriers for hydrophilic drugs.

DEPR:
The antibiotic compositions may, if desired, be presented in a pack or dispenser device which may contain one or more unit dosage forms containing the active ingredient. The pack may for example comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration.

DEPR:
The pharmaceutical compositions of the present invention comprise an antibiotic compound as the active ingredient, or a pharmaceutically acceptable salt thereof, and may also contain a pharmaceutically acceptable carrier, and optionally, other therapeutic ingredients, for example antivirals. The term "pharmaceutically acceptable salts" refers to salts prepared from pharmaceutically acceptable non-toxic acids and bases, including inorganic and organic acids and bases.

DEPR:
The pharmaceutical compositions include compositions suitable for oral, rectal, mucosal routes, transdermal, parenteral (including subcutaneous, intramuscular, intrathecal and intravenous), although the most suitable route in any given case will depend on the nature and severity of the condition being treated.

DEPR:
In practical use, an antibiotic agent can be combined as the active ingredient in intimate admixture with a pharmaceutical carrier according to conventional pharmaceutical compounding techniques. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g., oral or parenteral (including tablets, capsules, powders, intravenous injections or infusions). In preparing the compositions for oral dosage form any of the usual pharmaceutical media may be employed, e.g., water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, and the like; in the case of oral liquid preparations, e.g., suspensions, solutions, elixirs, liposomes and aerosols; starches, sugars, micro-crystalline cellulose, diluents, granulating agents, lubricants, binders, disintegrating agents, and the like in the case of oral solid preparations e.g., powders, capsules, and tablets. In preparing the compositions for parenteral dosage form, such as intravenous injection or infusion, similar pharmaceutical media may be employed, e.g., water, glycols, oils, buffers, sugar, preservatives and the like know to those skilled in the art. Examples of such parenteral compositions include, but are not limited to Dextrose

5% w/v, normal saline or other solutions.

DEPR:
For administration to subjects, antibiotic compounds discovered by using the assays of the invention are formulated in pharmaceutically acceptable compositions. The compositions can be used alone or in combination with one another, or in combination with other therapeutic or diagnostic agents. These compositions can be utilized in vivo, ordinarily in a mammal, preferably in a human, or in vitro. In employing them in vivo, the compositions can be administered to the mammal in a variety of ways, including parenterally, intravenously, subcutaneously, intramuscularly, colonially, rectally, vaginally, nasally, orally, transdermally, topically, ocularly, or intraperitoneally.

DEPR:
As will be readily apparent to one skilled in the art, the magnitude of a therapeutic dose of an antibiotic compound in the acute or chronic management of an infectious disease will vary with the severity of the condition to be treated, the particular composition employed, and the route of administration. The dose, and perhaps dose frequency, will also vary according to the species of the animal, the age, body weight, condition and response of the individual subject. The determination of effective dosage levels, that is the dosage levels necessary to achieve the desired result, will be within the ambit of one skilled in the art.

DEPR:
In selected cases, drug delivery vehicles may be employed for systemic or topical administration. They can be designated to serve as a slow release reservoir, or to deliver their contents directly to the target cell. Such vehicles have been shown to also increase the circulation half-life of drugs which would otherwise be rapidly cleared from the blood stream. Some examples of such specialized drug delivery vehicles which fall into this category are liposomes, hydrogels, cyclodextrins, and bioadhesive microspheres. These vehicles have been developed for chemotherapeutic agents.